Histone deacetylase inhibitor BL1521 induces a G1-phase arrest in neuroblastoma cells through altered expression of cell cycle proteins

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Abstract Histone deacetylase inhibitors (HDACi) have been discovered as potential drugs for cancer treatment. The effect of BL1521, a novel HDACi, on the cell cycle distribution and the induction of apoptosis was investigated in a panel of *MYCN* single copy and *MYCN* amplified neuroblastoma cell lines. BL1521 arrested neuroblastoma cells in the G1 phase and induced up to 30% apoptosis. Downregulation of CDK4, upregulation of $p21^{WAF1/CIP1}$ and an increase of hypophosphorylated retinoblastoma protein were observed, indicating a possible mechanism for the cell-cycle arrest. BL1521 also induced downregulation of p27, which may underlie the observed induction of apoptosis.

Keywords: Histone deacetylase inhibitor; G1 arrest; Apoptosis; Cyclin dependent kinase inhibitor; Cell cycle

1. Introduction

Neuroblastoma is a common childhood tumor originating from neural crest cells in the sympathetic nervous system and is responsible for over 15% of cancer-related death in children [1,2]. The clinical diversity of neuroblastoma correlates with several characteristic genetic features [1]. One of the best genetic markers of poor prognosis is the amplification of the *MYCN* gene. These tumors do not respond as well to the treatment used in the clinic today as compared to those without *MYCN* amplification, consequently leading to a disease related mortality of up to 70% [1,3]. Therefore, the development of new therapeutic strategies is warranted.

Histone deacetylase (HDAC) has recently been discovered as a potential target for the development of new therapeutic agents [4]. Deacetylation of histones results in a tightened chromatin structure and reduces the accessibility of DNA, leading to a decrease in transcriptional activity [5,6]. When HDAC function is inhibited, histones remain acetylated, resulting in a more open chromatin conformation, which facilitates the transcription of genes [6]. It has been shown that in a variety of tumor cells, these changes resulted in a reduction of proliferation and metabolic activity and in an induction of apoptosis and differentiation, both in vitro and in vivo [4].

Thus far, a number of histone deacetylase inhibitor (HDACi) have been identified with different anti-tumor activity, specificity, toxicity, and stability [4,6]. To date, conflicting results exists as to the effect of HDACi on cell cycle distribution. In a majority of tumor cells, HDACi appeared to upregulate the expression of $p21^{WAF1/CIP1}$ and downregulate cyclin D1, which was paralleled by a cell cycle arrest in G1 phase. However, downregulation of cyclin B1 together with a G2/M arrest has also been observed in response to HDACi treatment in tumor cells [7,8].

Treatment of neuroblastoma cells with a recently developed HDACi, BL1521 resulted in a significant decrease in cell proliferation, metabolic activity, and *MYCN* expression [9]. To date, the underlying mechanism for these observed effects is not known. Therefore, we have investigated the effect of BL1521 on the cell cycle distribution and the expression of cell cycle regulating proteins in a panel of neuroblastoma cell lines with different *MYCN* status.

2. Materials and methods

2.1. Cell culture

Three *MYCN* single copy neuroblastoma cell lines, SKNAS, SKNSH, and GIMEN, and three *MYCN* amplified neuroblastoma cell lines, SJNB8, SKNBE(2), and IMR32, were cultured in RPMI 1640 supplemented with 10% (v/v) heat inactivated fetal bovine serum, 50 U/ml penicillin/streptomycin, 0.2 mg/ml gentamycin, 0.25 μg/ml fungizone, and 4 mM glutamine. Cells were cultured at 37 °C in a 90% humidified atmosphere under 6.0% CO_2. All incubations of cells with compounds were carried out by preparing a 1:1000 dilution from the DMSO stock solutions in culture medium directly before use.

2.2. Flowcytometry

The percentage of cells in G1, S, and G2-M phase of the cell cycle was determined by flowcytometry using a bromodeoxyuridine (BrdU) and propidium iodide (PI) DNA staining procedure [10]. The stained

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Abbreviations: HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; TSA, trichostatin A; CDK, cyclin dependent kinase; RT, room temperature; HRP, horseradish peroxidase; Rb; retinoblastoma protein

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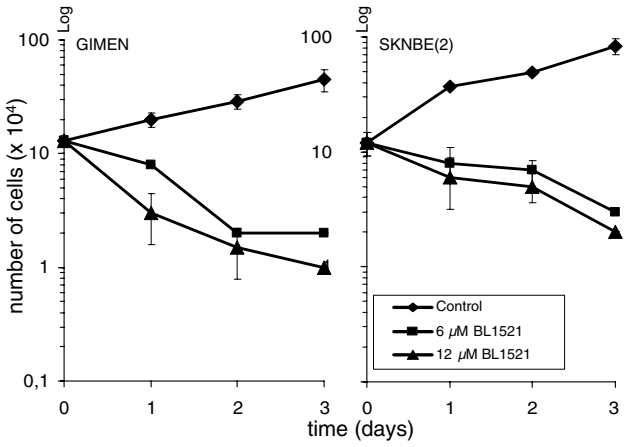

Fig. 1. Effects of BL1521 on the number of viable neuroblastoma cells. Growth curve analysis of *MYCN* single copy (GIMEN) and *MYCN* amplified (SKNBE(2)) cells was determined by counting the cells following treatment with either 6 or 12 μM BL1521. Data represent the average of four independent experiments and includes standard deviations.

cell nuclei were run on a FACS calibur (Beckton–Dickinson) flowcytometer and the data were subsequently analyzed using the WinMD1 version 2.7 software.

2.3. Staining of apoptotic cells with bis-benzimide (Hoechst, 33342)

The nuclear fragmentation of apoptotic cells was assessed by staining the cells with the DNA fluorochrome bis-benzimide (Hoechst, 33342). Cells were grown in polystyrene slide flasks. After treatment of the cells with BL1521, they were fixed by adding 3 ml of a 1:3 methanol–acetic acid solution to the medium (3 ml) and incubated for 5 min at room temperature (RT), followed by replacing the mixture with pure 1:3 methanol–acetic acid twice. The fixed cells were air dried and incubated with 10 mg/ml bis-benzimide (Sigma) in PBS at RT for 20 min. Subsequently, the cells were washed three times in MilliQ after which the cells were examined using a fluorescence microscope (Zeiss), filter 395–440, FT 460, LP 470. Cells with condensed chromatin or fragmented nuclei were considered to be apoptotic.

2.4. Trypan blue dye exclusion test

The loss of membrane integrity in dying cells allows the preferential uptake of trypan blue. Cells were harvested using 1× trypsin–EDTA (Sigma) in PBS. Thereafter, 10 μl of the suspended cells was mixed with 10 μl of 0.4% (w/v) trypan blue in PBS, and examined using a Bürkerturk counting frame.

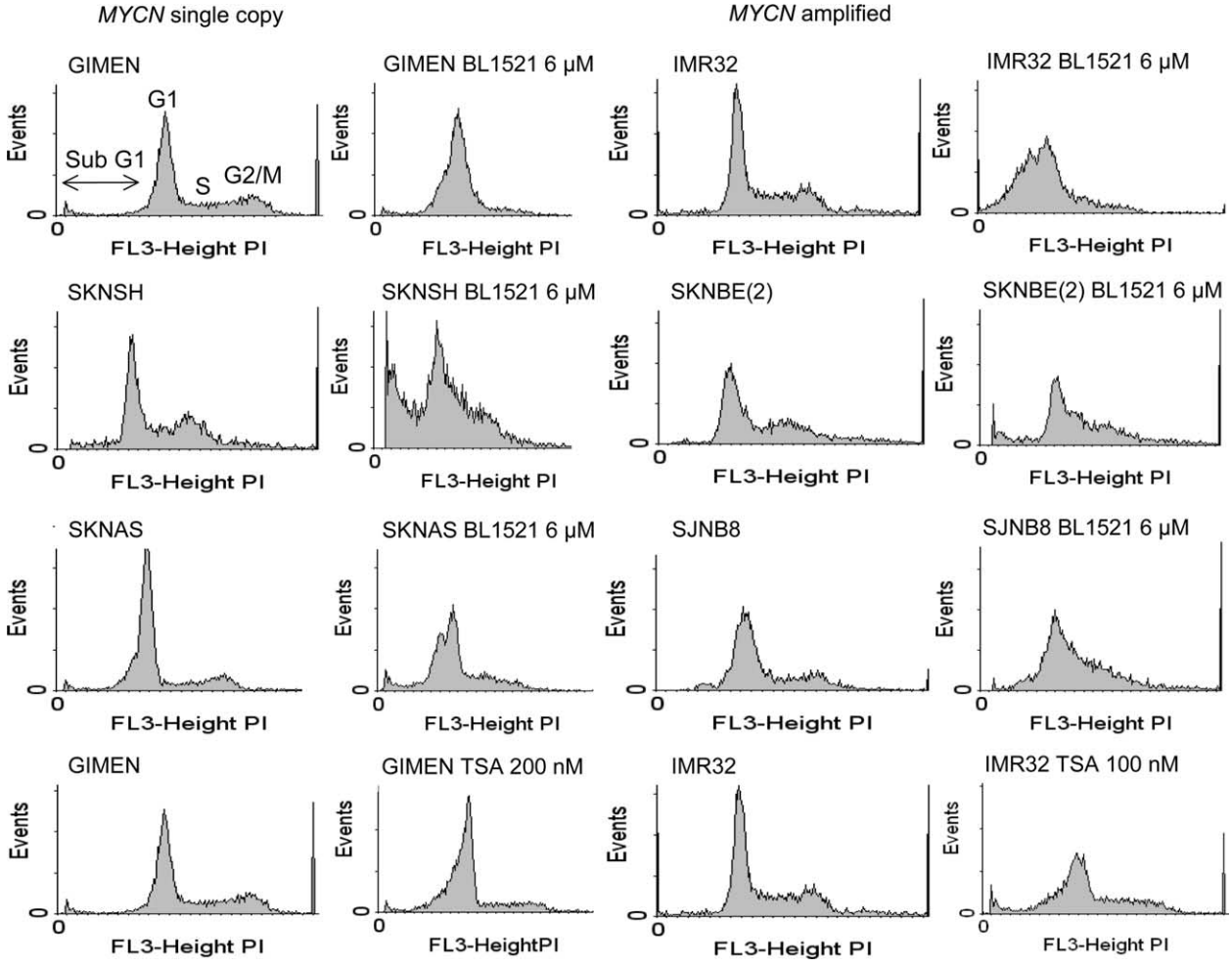

Fig. 2. Univariate analysis of cell cycle distribution of neuroblastoma cells with or without amplified *MYCN*, treated with 6 μM BL1521 for 24 h. The number of cells is plotted against PI-fluorescence as a measure of DNA content. In the top left figure, the G1, S, and G2/M phases of the cell cycle are depicted and the sub-G1 region, which is indicative of apoptosis. TSA was included as a positive control for G1 arrest by HDACi. Representative examples are shown of three separate experiments.

2.5. Western analysis

Expression levels of proteins were determined by western analysis, according to the methods used in Ref. [9]. Rabbit polyclonal antibodies against CDK4 (Santa Cruz) and p27 (AbCam) were used in combination with swine-anti-rabbit polyclonal antibody, conjugated to horseradish peroxidase (HRP) (Dako Cytomation). Mouse monoclonal antibodies against $p21^{WAF1/CIP1}$ and retinoblastoma protein (Rb, BD Pharming Gen) were used in combination with rabbit-anti-mouse polyclonal antibodies, conjugated to HRP (Biorad for $p21^{waf1/cip1}$ detection and Dako Cytomation for Rb detection). The signal was detected with ECL+ (Amersham Bioscience) and Bio-Max light film (Kodak). Equal loading of the gels was determined using Ponceau staining.

3. Results

To quantify the anti-proliferative effects of BL1521 in neuroblastoma cells, the cell panel was cultured with different concentrations of BL1521 for 1, 2, and 3 days. Representative examples after BL1521 treatment for *MYCN* single copy and *MYCN* amplified neuroblastoma cells are shown in Fig. 1. A significant time-dependent decrease in cell number was observed in response to incubation with BL1521, demonstrating that BL1521 was cytotoxic for the neuroblastoma cells. A 3-day incubation with 12 μM BL1521 resulted in a 92% reduction of the number of viable GIMEN cells and a 83% reduction of the number of viable SKNBE(2) cells compared to the untreated cells.

The effect of BL1521 on the cell cycle of neuroblastoma cells is shown in Fig. 2. Upon treatment of either single copy or amplified *MYCN* cells with BL1521, the cell cycle distribution of the cells, was markedly changed. The amount of cells in S phase as well as the G2/M phase decreased, whereas the amount of cells in the sub-G1 and G1 phase showed a substantial increase. The cells in the sub-G1 were identified as dead cells.

To quantify the amount of S phase cells, BrdU incorporation studies were performed. After 24 h of BL1521 treatment, neuroblastoma cells exhibited a marked decrease of the amount of cells in S phase and G2/M phase paralleled by an increase of cells in G1 phase. Representative results are shown in Fig. 3: in response to BL1521 SKNAS and IMR32 cells showed a reduction of S phase of 22.9% and 43.2%, respectively. Interestingly, part of the S-phase cells did not incorporate BrdU and these cells were therefore defined as dying or dead cells according to Ref. [11].

To elucidate the mechanism behind the induction of G1 phase arrest by BL1521, we studied the change in concentration of the cyclin dependent kinase (CDK) inhibitors $p21^{WAF1/CIP1}$ and p27, CDK4, and the change in Rb phosphorylation status in neuroblastoma cells (Fig. 4). The amount of the $p21^{WAF1/CIP1}$ protein increased in response to treatment with BL1521 as was expected from our previous studies [9]. In contrast, the protein concentration of p27 had decreased upon BL1521 treatment. This decrease of p27 was more substantial for the *MYCN* amplified cell line, compared to the *MYCN* single copy cell line. Furthermore, the complex partner of cyclin D, CDK4, also showed reduced expression in response to BL1521 treatment. In line with the increased $p21^{WAF1/CIP1}$ level and the decreased CDK4 level, an increase in the hypophosphorylated state of Rb was observed.

To quantify the amount of apoptosis induced by BL1521, nuclear fragmentation was determined. Fig. 5A shows normal

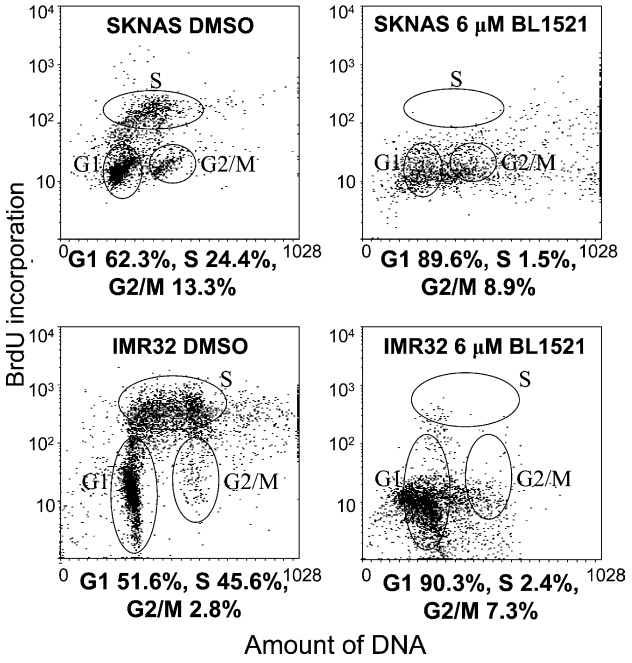

Fig. 3. Bivariate flowcytometry with *MYCN* single copy (SKNAS) and *MYCN* amplified (IMR32) neuroblastoma cells, treated with BL1521 compared to control. Representative results of four independent experiments are shown, with a SD ± 8. Similar results were found in other single copy and amplified *MYCN* cell lines.

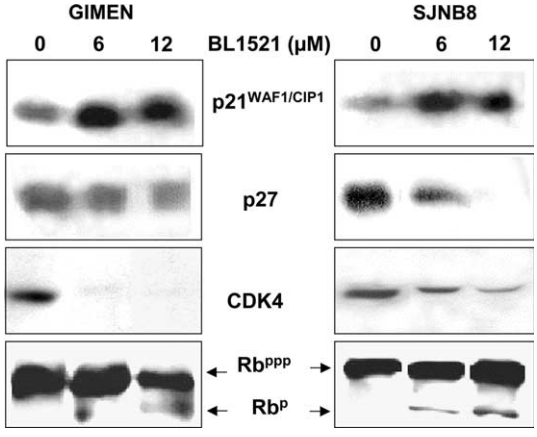

Fig. 4. Western blot analysis of $p21^{WAF1/CIP1}$, CDK4, Rb, and p27 protein. Cells were incubated for 24 h with either 6 or 12 μM BL1521. Results were shown for neuroblastoma cells with amplified *MYCN* status (SJNB8) and single copy *MYCN* status (GIMEN).

cell nuclei of untreated cells and fragmented cell nuclei after 24 h of treatment with BL1521. The number of cells displaying these fragmented cell nuclei increased in a dose responsive fashion as is shown in Fig. 5B. The *MYCN* amplified neuroblastoma cell line SKNBE(2) showed a significant increase ($P < 0.05$) in the number of apoptotic cells from 1.9% ± 0.5 for control cells, after treatment with 6 μM BL1521 15.9% ± 8.0 up to 31.8% (±12.8) after treatment with 12 μM of BL1521. The *MYCN* single copy cell line, GIMEN, appeared to be less sensitive to the induction of apoptosis by BL1521, resulting in a maximal induction of apoptosis of 9.7% ± 6.9 after treatment with 12 μM of BL1521.

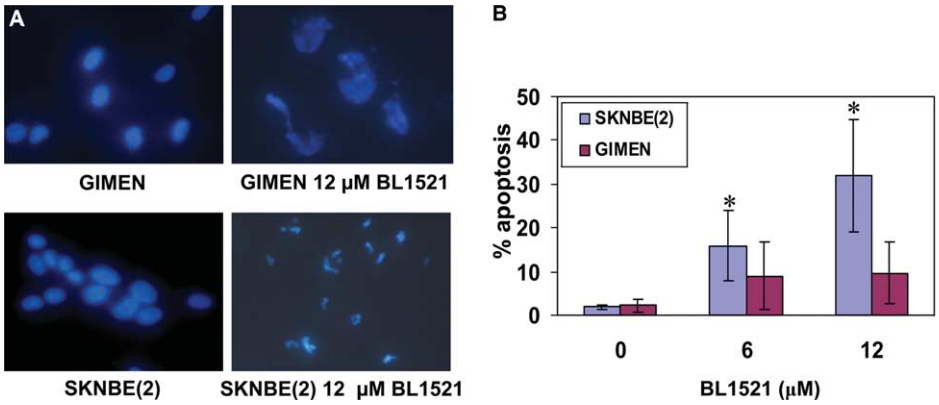

Fig. 5. (A) The nuclear staining of *MYCN* single copy neuroblastoma cells (GIMEN) and *MYCN* amplified neuroblastoma cells (SKNBE(2)) with Hoechst. On the left control cells were shown, whereas on the right cells treated with 12 μM BL1521 for 24 h. (B) The percentage of fragmented cell nuclei of neuroblastoma cells, with (SKNBE(2)) or without (GIMEN) amplified *MYCN* status, treated for 24 h with either 6 or 12 μM BL1521. Data represent the average of three independent experiments and includes standard deviations. *Significant difference compared to control ($P < 0.05$ using t test).

4. Discussion

In this study, we investigated the underlying mechanism for the observed decrease in proliferation found in neuroblastoma cells in response to incubation with the HDACi BL1521. Therefore, we determined the effects of BL1521 on the cell cycle and quantified the amount of apoptosis. Our results showed that BL1521 induced both an arrest in the G1 phase of the cell cycle and an increase in the number of apoptotic cells. Several mechanisms are able to induce the observed G1 arrest, as depicted in Fig. 6. We identified four different possibilities which will be discussed in more detail in the following sections.

The induction of G1 arrest could be resulting from the upregulation of the members of the CIP/KIP family of cyclin–CDK inhibitors $p21^{WAF1/CIP1}$, p27, and p57. $p21^{WAF1/CIP1}$ is able to reduce the progression through the G1 phase and transition into the S phase in cells through, on the one hand, activating the restriction-point of the cell cycle and on the other hand, the inhibition of CDK2 and cyclin D–CDK4/6 complexes [12–15]. Cyclin–CDK complex activity is necessary for the progression of the cell cycle through G1-S and G2-M (Fig. 6) [12,14]. When the restriction-point is activated no transition into S phase can take place and cells become more prone to apoptosis [16].

It has been shown that a number of HDACi are able to increase the expression of $p21^{WAF1/CIP1}$ [17–20]. We previously showed that BL1521 treatment of neuroblastoma cells resulted in an increase of $p21^{WAF1/CIP1}$ mRNA [9]. This study showed that the concentration of $p21^{WAF1/CIP1}$ protein increased as well. Although it has been reported that in some neuroblastoma cell lines the endogenous $p21^{WAF1/CIP1}$ is dysfunctional, the upregulation of $p21^{WAF1/CIP1}$ may still be partly responsible for the observed G1 phase arrest [21]. Unexpectedly the concentration of the p27 protein decreased in response to treatment with BL1521. Recently, it was described that p27 knock out cells were more sensitive to apoptotic stimuli than wild-type p27 cells and that a high concentration of p27 in the cytoplasm favor the survival under conditions, which normally induce apoptosis [22,23]. Since we observed that *MYCN*

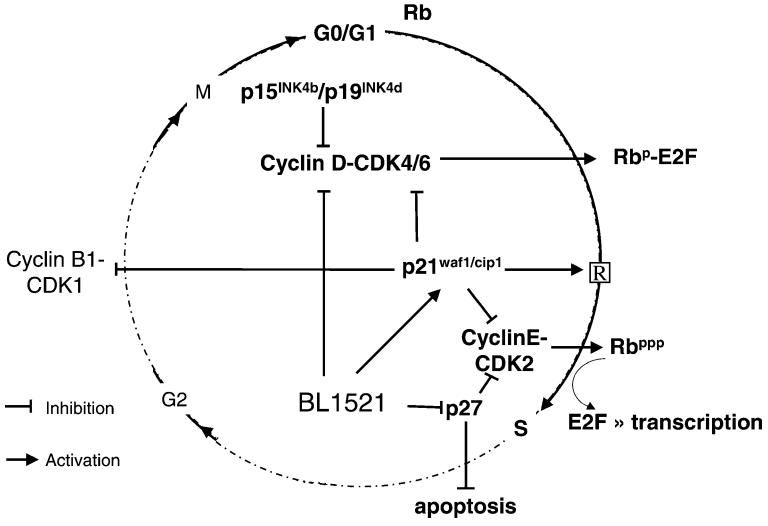

Fig. 6. Regulation of the G1-S transition by the cyclin–CDK/Rb pathway during BL1521 treatment [12,24].

amplified cells were more prone to apoptosis and showed a more substantial decrease of p27, in comparison with *MYCN* single copy cells, the induction of apoptosis, as a result of BL1521 treatment is in line with the downregulation of p27.

The next possible contribution to the observed BL1521 induced G1 arrest could be the altered presence of members of the cyclin D–CDK4/6 complex. The cyclin D–CDK4 complex is responsible for the hyperphosphorylation of under-phosphorylated Rb. Under-phosphorylated Rb binds members of the E2F family of transcription factor rendering them inactive. Consequently, hyperphosphorylation of Rb results in the release of the E2F transcription factors leading to the expression of cyclin E and thus transition into the S phase of the cell cycle (Fig. 6) [4,12,14,24]. In our experiments, we observed a downregulation of CDK4 protein in response to BL1521. Accordingly, we observed that the concentration of hypophosphorylated form of the Rb protein increased, suggesting that the downregulation of CDK4 may also be partly responsible for the observed G1 phase arrest in response to BL1521.

The third possible factor that could contribute to the induction of G1 phase arrest in response to BL1521 incubation is the upregulation of the INK4 family members. Under particular conditions HDACi have been reported to be able to upregulate $p15^{INK4b}$ and $p19^{INK4d}$ expression [25,26]. The $p15^{INK4b}$ and $p19^{INK4d}$ proteins function through the inhibition of the activity of the cyclin D–CDK4/6 complex [12,14,25,26]. However, we could not detect any $p19^{INK4d}$ or $p15^{INK4b}$, which might be explained by the high incidence of deletion of the $p15^{INK4b}$ locus, 9p21, and loss of heterozygozity of 9p in neuroblastoma that have been reported, resulting in loss of expression [27,28].

A fourth possible factor that should be taken into consideration is the effect of *MYCN* on the activation of the cyclin D–CDK4/6 complexes and on the downregulation of members of the INK4 and CIP/KIP family. These effects of *MYCN* enhance S phase progression and partly explain the high proliferative activity of *MYCN* amplified neuroblastoma cells [29,30]. Our previous results showed that *MYCN* expression had been downregulated in response to BL1521, possibly contributing to the induction of the G1 arrest [9]. However, Fig. 2 shows that the G1 phase arrest, induced by BL1521 in both the *MYCN* amplified and the *MYCN* single copy neuroblastoma cells, takes place in an approximately similar fashion. This suggests that the expression of *MYCN*, is not a major factor in establishment the G1 phase arrest.

Apart from the ability to induce cell cycle arrest, HDACi are known to be able to induce apoptosis [9,31]. Previously, we showed that BL1521 is able to induce PARP cleavage, an end stage marker of apoptosis, in a panel of neuroblastoma cell lines [9]. Our results showed that *MYCN* amplified cell lines appear to be more sensitive to the induction of apoptosis by BL1521 than the *MYCN* single copy cell lines. We have already discussed the possible role of p27 in this difference in sensibility. Moreover, apart from the proliferative action of *MYCN*, it also has a function in promoting apoptosis suggesting a second reason for the difference in sensitivity towards undergoing apoptosis [32]. A third optional mechanism for the induction of apoptosis in response to BL1521 could be the increased ratio between pro-apoptotic and anti-apoptotic genes [33]. Currently, efforts are being made to elucidate the mechanism of apoptosis in neuroblastoma cells in response to BL1521 treatment.

In conclusion, these results show that the decrease in proliferation observed in neuroblastoma cells in response to BL1521 treatment is a concerted result of the induction of apoptosis and the induction of G1 phase arrest executed via the upregulation of $p21^{WAF1/CIP1}$ and or downregulation of CDK4.

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